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Spinal NKCC1 blockade inhibits TRPV1-dependent referred allodynia

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Abstract

Background: The Na^+, K^+, $2Cl^-$ type I cotransporter (NKCC1) and TRPV1 receptors, at the level of the dorsal horn, have been implicated in mediating allodynia in response to an inflammatory insult. The NKCC1 cotransporter regulates intracellular [Cl] and thus the magnitude and polarity of $GABA_A$ receptor responses in neurons. TRPV1 receptors transduce diverse chemical and natural stimuli in nociceptors and are critical for inflammatory hyperalgesia.

Results: Here we have tested the role of spinal NKCC1 cotransporters and TRPV1 receptors in referred allodynia in a model of visceral hyperalgesia in mice. Intrathecal (IT) injection of the NKCC1 inhibitor bumetanide (BUM, 1 nmol) inhibited referred, abdominal allodynia evoked by an intracolonic capsaicin injection. BUM was effective when injected IT either before or up to 4 hrs after the establishment of referred allodynia. The TRPV1 antagonist AMG 9810 (1 nmol) also inhibited referred allodynia in this model suggesting the involvement of an endogenous TRPV1 agonist in the dorsal horn in referred allodynia. In support of this suggestion, the endovanilloid TRPV1 agonist, narachidonoyl- dopamine (NADA, 1 or 10 nmol, IT) evoked stroking allodynia in the hindpaw that was blocked by co-treatment with AMG 9810 (1 nmol). The TRPV1-dependent stroking allodynia caused by NADA appeared to be functionally linked to NKCC1 because BUM (1 nmol) also inhibited NADA-evoked stroking allodynia.

Conclusion: Our findings indicate that spinal NKCC1 and TRPV1 are critical for referred allodynia mediated by a painful visceral stimulus. Moreover, they suggest that endogenous TRPV1 agonists, released in the CNS in painful conditions, might stimulate TRPV1 receptors on primary afferents that, in turn, play a role in increasing NKCC1 activity leading to allodynia.

Background

Intracellular chloride concentration in neurons is maintained by members of the Na^+, K^+, $2Cl^-$ (NKCC) and K^+, Cl^- (KCC) families of cation-chloride cotransporters [1]. The NKCC proteins accumulate chloride intracellularly and, in dorsal root ganglion (DRG) neurons, it is the primary mechanism that sets the reversal potential for chloride conductance through $GABA_A$-receptors ($GABA_AR$)

[2,3]. Unlike most CNS neurons, DRG neurons maintain depolarizing responses to $GABA_AR$ agonists throughout postnatal development [2,3]. These depolarizing $GABA_AR$ responses are dependent on NKCC1 expression because depolarizing $GABA_AR$ responses in DRG neurons are reduced in NKCC1$^{-/-}$ mice [3]. It has been suggested that some pain states might involve enhancements of primary afferent $GABA_AR$ responses such that the normal small GABAergic epolarization of these fibers is augmented to the point that it induces a direct activation of spinal nociceptors [4-7]. This has led to the proposal that NKCC1 is responsible for the increase in intracellular chloride that could mediate $GABA_AR$-dependent depolarization above threshold for spike generation in nociceptors [5-7]. In support of this hypothesis, it has been shown that NKCC1$^{-/-}$ mice display reduced responses to noxious heat as well as reduced touch-evoked pain [3,8]. Furthermore, intrathecal delivery of the NKCC1 blocker bumetanide (BUM) inhibits nocifensive behavior in phase II of the formalin test [9] and mechanical allodynia induced by capsaicin injection into the hindpaw [10] in rats. Finally, intracolonic capsaicin injection stimulates a rapid and transient increase in spinal phosphorylated NKCC1 and a long lasting increase in trafficking of NKCC1 protein to the plasma membrane [11]. Taken together these findings indicate that NKCC1 might play an important role in inflammatory and tissue damage pain.

In naive animals, Aα-fiber stimulation causes a $GABA_AR$-dependent primary afferent depolarization (PAD) of nociceptors leading to a decrease in pain transmission in the spinal dorsal horn [7,12]. In inflammatory conditions Aβ-fibers are capable of directly exciting nociceptors via a GABAergic mechanism causing antidromic (termed dorsal root reflexes, DRRs) and orthodromic firing of nociceptors [13-16]. This process has been proposed as a mechanism of inflammation- or injury-evoked allodynia. Because it is dependent on depolarizing $GABA_AR$ responses, NKCC1 is a logical molecular candidate for mediating this effect [5-7]. Here we have tested the hypothesis that spinal NKCC1 mediates referred allodynia in response to a visceral inflammatory stimulus. TRPV1 receptors in the CNS, likely localized on primary afferent terminals in the dorsal horn, have recently been identified as an important target for inflammatory allodynia [17]. Hence, we have also tested the hypothesis that spinal TRPV1 receptors are involved in referred allodynia and we have investigated a possible link between spinal TRPV1-dependent allodynia and NKCC1. Our findings demonstrate that spinally applied inhibitors of NKCC1 and TRPV1 attenuate referred allodynia evoked by a painful visceral stimulus and show that spinally applied TRPV1 agonists cause allodynia that is likewise inhibited by NKCC1 blockade.

Results

Spinal NKCC1 blockade inhibits intracolonic capsaicin-evoked referred, abdominal allodynia and hyperalgesia

Work from this laboratory has shown previously that an intracolonic capsaicin injection causes a transient increase in NKCC1 phosphorylation and a sustained increase in plasma membrane localization of NKCC1 in the spinal dorsal horn [11]. Here we have tested the hypothesis that NKCC1 is functionally linked to referred allodynia and hyperalgesia in this model using spinal application of the NKCC1 inhibitor bumetanide (BUM). We first sought to determine the effects of increasing doses of intrathecal (IT) BUM by itself and the effects of IT BUM on referred (abdominal) allodynia and hyperalgesia 0.5 hrs following an intracolonic capsaicin (0.1%) injection. In this model referred allodynia peaks at 20 min post capsaicin injection, is sustained at this peak level for 6 hrs, and referred allodynia is evident for at least 24 hrs [18,19]. BUM at 0.1, 1 and 10 nmol had no effect on response frequencies to 1 (Fig 1A) or 32 mN (Fig 1B) stimulation of the abdomen. When BUM, at the same doses, was injected IT 5 min prior to intracolonic capsaicin it dose-dependently inhibited referred allodynia (1 mN stimulation of the abdomen, Fig 1C) and 1 nmol BUM inhibited referred hyperalgesia (32 mN stimulation of the abdomen, Fig 1D). BUM (1 nmol) had no effect on nocifensive responses evoked by intracolonic capsaicin (VEH = 88.0 ± 10.7 s, BUM = 78.5 ± 13.6 s) nor did it influence the latency to first nocifensive behavior (VEH = 77.7 ± 17.4 s, BUM = 57.8 ± 12.9 s).

Next we addressed the time course of IT BUM effects on the inhibition of intracolonic capsaicin-evoked referred allodynia. BUM (1 nmol) was injected IT 5 min prior to intracolonic capsaicin application and significantly inhibited responses to 1 mN abdominal stimulation at 0.5 hrs (Fig 2A). However, BUM injected mice developed referred allodynia by 1 hr and this was maintained at 3 hrs illustrating that, while spinal NKCC1 blockade inhibits referred allodynia, it does not prevent its subsequent development (Fig 2A). If spinal NKCC1 activity contributes to the maintenance of referred allodynia we would predict that IT BUM injection following intracolonic capsaicin would inhibit abdominal allodynia. When BUM (1 nmol) was injected IT 20 min following intracolonic capsaicin it inhibited referred allodynia 0.5 and 1 hr post capsaicin (Fig 2B). Three hrs following intracolonic capsaicin the BUM group was not different from baseline (did not display referred allodynia) but was also not different from mice that received IT VEH. When BUM (1 nmol) was injected IT 4 hrs post capsaicin it inhibited referred allodynia 0.5 and 1 hr post BUM injection (Fig 2C). At the 7 hr time point the BUM group was not statistically different from baseline (did not display referred allodynia) but was also not different from the IT VEH group. These findings indicate that spinal NKCC1 blockade either before or after

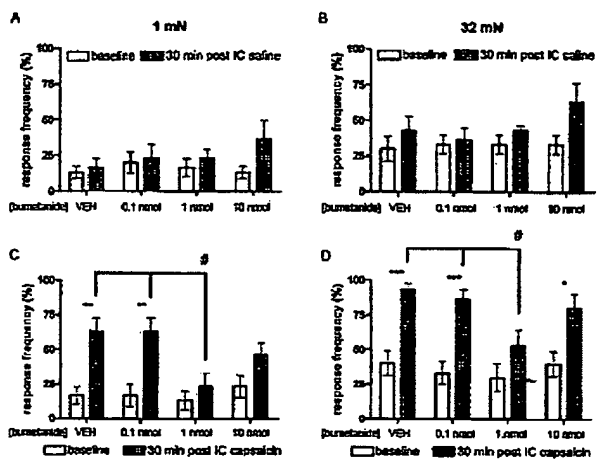

Figure 1
NKCC1 inhibitor bumetanide blocks intracolonic capsaicin-evoked referred allodynia and hyperalgesia. Three doses of bumetanide or vehicle (VEH) were injected intrathecally (IT) and saline or capsaicin was injected into the colon 5 min later. A and B) Baseline response frequencies to abdominal 1 mN and 32 mN von Frey hair application are shown as well as 30 min after the intracolonic (IC) saline injection. C and D) Response frequencies to abdominal 1 mN (C) and 32 mN (D) von Frey hair application 30 min after the IC capsaicin injection are shown in comparison to baseline von Frey response frequencies. Stars (*) indicate significant effects vs. baseline (two-way anova); number signs (#) indicate significant effects of IT bumetanide injections (one-way anova, # or * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 6 per group).

intracolonic capsaicin is capable of inhibiting referred allodynia.

Spinal TRPV1 receptors regulate referred allodynia: role of NKCC1

The role of TRPV1 in inflammatory hyperalgesia is well established and appears to involve a peripheral mechanism in which TRPV1 receptors are sensitized and respond to lower temperature stimulation than their basal thresholds [20,21]. It is also known that TRPV1 is involved in inflammatory mechanical hyperalgesia and, interestingly, recent studies have suggested that this effect is due to TRPV1 receptors in the CNS, likely on primary afferents in the dorsal horn [17]. Hence, we hypothesized that spinal TRPV1 receptors might be involved in referred allodynia in response to intracolonic capsaicin. The TRPV1 antagonist AMG 9810 (1 nmol) [22] was injected IT 5 min prior to intracolonic capsaicin and referred allodynia (1 mN stimulation of the abdomen) was measured at 0.5, 1.0 and 2.0 hrs. Mice that received an IT AMG 9810 injection did not differ from baseline at 0.5, 1.0 or 2.0 hrs post intracolonic capsaicin injection while mice that received an IT VEH injection displayed referred allodynia at all time points (Fig 3). The AMG 9810 group was significantly decreased vs. VEH injected animals at the 0.5 hr time point but not at 1.0 and 2.0 hrs (Fig 3). This observation illustrates that spinal TRPV1 receptors are involved in referred allodynia evoked by an intracolonic capsaicin injection.

The previous findings cited above and our present observation with AMG 9810 suggests that an endogenous TRPV1 agonist is active in the spinal dorsal horn and is involved in regulating mechanical nociceptive thresholds. One such candidate endogenous TRPV1 agonist is n-arachidonoyl-dopamine (NADA) [23]. NADA is a potent TRPV1 agonist, causes hyperalgesia in vivo [23,24] and exists in pharmacologically relevant concentrations in the CNS [23,25]. We hypothesized that IT NADA would stimulate allodynia and/or hyperalgesia in the hindpaw. 1 or 10 nmol NADA or VEH was injected IT and the hindpaw was stimulated with 1 - 16 mN von Frey hairs 15 and 60 min following the IT injection. 1 nmol NADA had no effect on von Frey response frequency at any time point and 10 nmol NADA evoked hyperalgesia to an 8 mN stimulus only at the 60 min time point (Fig 4A and 4B). We also measured stroking allodynia as measured by stroking the hindpaw with a cotton bud. Mice injected with 10 nmol NADA displayed stroking allodynia at both 15 and 60 min following IT NADA (Fig 4C and 4D). Mice injected with 1 nmol NADA had stroking allodynia only at the 60 min time point (Fig 4D). We did not observe nocifensive behaviors in response to NADA at either dose (data not shown). This finding establishes that the TRPV1 agonist NADA evokes stroking allodynia in the hindpaw following spinal application. We next tested the ability of the TRPV1 antagonist AMG 9810 (1 nmol) to block the effect of NADA (10 nmol). AMG 9810 completely blocked NADA-evoked stroking allodynia at 15 and 60 min post IT injection (Fig 5A and 5B) indicating that the effects of NADA are TRPV1-mediated.

Finally, we hypothesized that NADA-evoked stroking allodynia would be attenuated by the NKCC1 blocker BUM. When BUM (1 nmol) was co-injected with NADA stroking allodynia was completely blocked 15 min following IT injection (Fig 5C). Sixty min post injection BUM injected mice did not differ from VEH but response frequency to a stroking stimulus also did not differ from NADA injected animals (Fig 5D). Hence, the NKCC1 inhibitor BUM reduces spinally mediated TRPV1-dependent stroking allodynia.

Discussion

In naive animals Aβ-fiber stimulation inhibits C-fiber activity via a mechanism called primary afferent depolari-

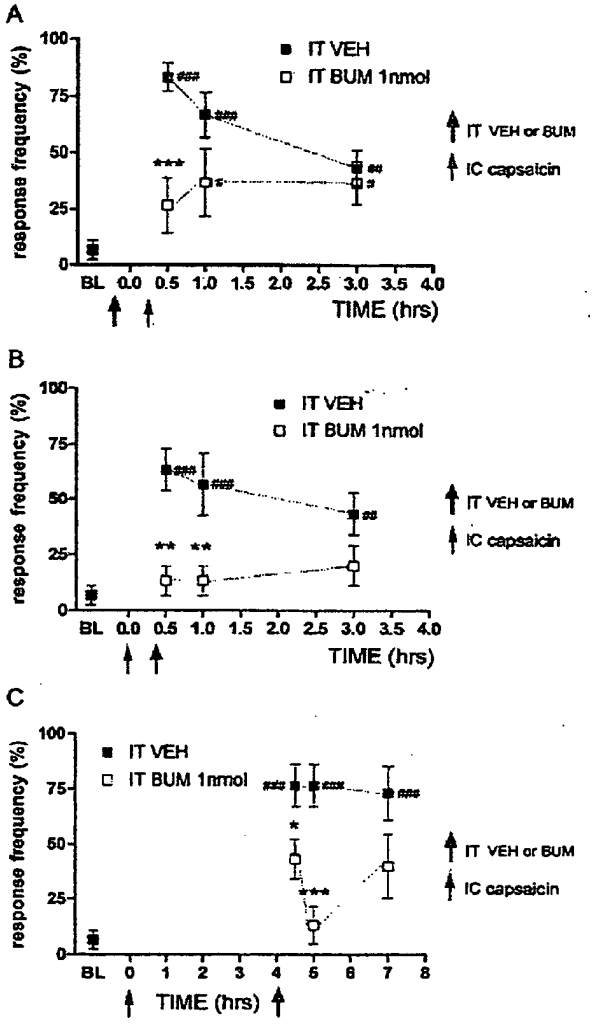

Figure 2
Time course of NKCC1 inhibitor bumetanide blockade of intracolonic capsaicin-evoked referred allodynia. Bumetanide (BUM) or vehicle (VEH) was injected intrathecally (IT) 5 min prior to intracolonic (IC) capsaicin (A) or 20 min (B) or 4 hrs (C) after IC capsaicin. Response frequencies to 1 mN von Frey hair application to the abdomen are shown at the indicated time points post IC capsaicin. Stars (*) indicate significant effects of BUM vs. VEH (two-way anova) while number signs (#) indicate significant effects vs. baseline (BL) at a given time point (one-way anova, # or * $p < 0.05$, ## or ** $p < 0.01$, ### or *** $p < 0.001$; n = 6 per group).

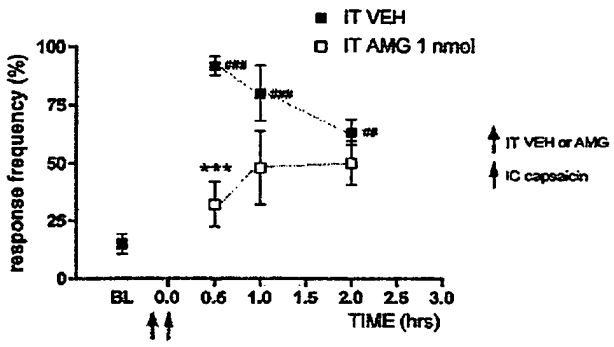

Figure 3
Spinal TRPV1 antagonism inhibits intracolonic capsaicin-evoked referred allodynia. The TRPV1 antagonist AMG 9810 (AMG, 1nmol) was injected intrathecally (IT) 5 min prior to intracolonic (IC) capsaicin. Response frequencies to abdominal von Frey hair (1 mN) stimulation at the indicated time points post intracolonic capsaicin are shown. Stars (*) indicate significant effects of AMG vs. VEH (two-way anova) while number signs (#) indicate significant effects vs. baseline (BL, one-way anova) at a given time point (## $p < 0.01$, ### or *** $p < 0.001$; n = 6 per group).

zation (PAD) [7,12]. PAD is mediated by $GABA_A$Rs on primary afferents where GABA has a depolarizing action. The molecular mechanism for depolarizing $GABA_A$R currents appears to be expression of NKCC1 in DRG neurons leading to a high intracellular Cl^- concentration and Cl^- efflux through the $GABA_A$R channel upon pharmacological stimulation [2,3]. We and others have proposed that GABAergic mechanisms may explain allodynia in inflamed conditions such that NKCC1 activity increases leading to a large depolarization in C-fibers, causing spiking, in response to Aβ-fiber stimulation [5-7]. Several lines of evidence support this claim: 1) following an inflammatory stimulus, lamina I neurons acquire a novel Aβ-fiber input that is GABAergic in nature [13], 2) stroking allodynia following capsaicin injection into the hindpaw is absent in NKCC1 knockout mice [8], 3) spinal application of the NKCC1 blocker BUM inhibits intraplantar capsaicin-evoked, secondary mechanical allodynia in rats [10] and 4) a painful visceral stimulus increases dorsal horn NKCC1 phosphorylation and membrane trafficking [11], both of which are mechanisms known to increase NKCC1 activity and intracellular Cl^- concentrations [1,26]. BUM also inhibits formalin induced nocifensive behaviors in the second phase of this model through both a spinal and peripheral mechanism [9] although the relevance of these findings to allodynia are not clear. Here we have added to this line of evidence by demonstrating that spinal application of BUM inhibits referred allodynia evoked by capsaicin injection into the colon. Taken together, these observations indicate that NKCC1 is an important regulator of touch-evoked pain in inflammatory conditions and that small molecules that inhibit this

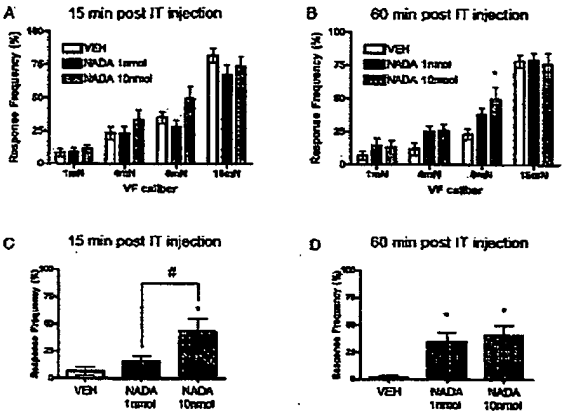

Figure 4
Spinal TRPV1 agonist causes stroking allodynia in the hindpaw. The endogenous TRPV1 agonist NADA (1 or 10 nmol) or vehicle (VEH) was injected intrathecally (IT) and the hindpaws were stimulated with von Frey hairs of the indicated calibers 15 (A) and 60 min (B) or a cotton bud 15 (C) and 60 min (D) after the IT injection. Stars (*) above columns indicate significant effects of NADA vs. VEH while number signs (#) above horizontal bars indicate significant effects of NADA 1 nmol vs. NADA 10 nmol (# or * $p < 0.05$; n = 10 per group). Panels A and B, two-way anova; panels C and D one-way anova.

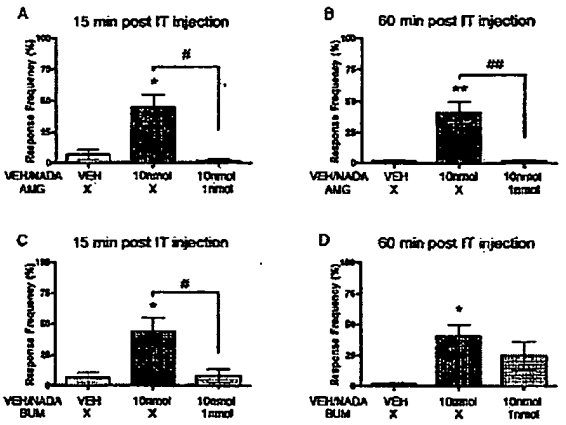

Figure 5
NADA-evoked stroking allodynia is TRPV1-dependent and inhibited by bumetanide. The endogenous TRPV1 agonist NADA (10 nmol) or vehicle (VEH) was injected intrathecally (IT) with or without a co-injection of the TRPV1 antagonist AMG 9810 (1 nmol) and the hindpaws were stimulated with a cotton bud 15 (A) and 60 min (B) after the IT injection. NADA (10 nmol) or VEH was injected IT with or without a co-injection of the NKCC1 antagonist bumetanide (BUM, 1 nmol) and the hindpaws were stimulated with a cotton bud 15 (C) and 60 min (D) after the IT injection. Response frequencies to the cotton bud stimulation are shown. Stars (*) above columns indicate significant effects of NADA vs. VEH while number signs (#) above horizontal bars indicate significant effects of antagonists (one-way anova, * or # $p < 0.05$, ** or ## $p < 0.01$; n = 6 per group).

cotransporter might have clinical utility for allodynia in humans.

Some pharmacological considerations deserve attention with respect to IT BUM. The anti-hyperalgesic (32 mN abdominal stimulation) effect of BUM was limited to a narrow dose range (1 nmol). This is expected because BUM possesses only roughly a 10–100 fold selectivity for NKCC1 vs. KCC2 [27]. Inhibition of KCC2 by BUM would be expected to have the opposite effect on touch-evoked responses because decreased KCC2 expression (analogous to pharmacological inhibition) or KCC2 blockade with DIOA reverses the polarity of $GABA_AR$ responses in a subset of lamina I and II neurons, leading to allodynia which has been proposed as a mechanism of neuropathic pain [28]. KCC2 is not expressed by DRG neurons [29] so this consideration applies only to intrinsic dorsal horn neurons. We focused on the 1 nmol dose in the rest of the experiments because of the KCC2 consideration stated above. While pretreatment with BUM inhibited referred allodynia it did not block its development. This finding likely reflects the long-lasting nociceptive stimulus evoked by intracolonic capsaicin [19] and illustrates that inhibition of NKCC1 is not sufficient to block the development of referred allodynia. On the other hand, BUM did effectively inhibit referred allodynia after its establishment both at 20 min and 4 hrs post intracolonic capsaicin injection suggesting that NKCC1 inhibitors might be clinically useful in cases of established inflammatory allodynia.

The TRPV1 receptor is expressed by a subset of DRG neurons that project to lamina I and II of the dorsal horn [30]. The majority of these neurons also express NKCC1 [29], hence, we reasoned that spinal TRPV1 receptors might be involved in regulating NKCC1 activity. The role of TRPV1 receptors in thermal hyperalgesia is well established [20,21] and, recently, it has become clear that spinal TRPV1 receptors play a role in allodynia [17]. Our findings show that spinal application of AMG 9810, a potent and highly selective TRPV1 antagonist, inhibits referred allodynia in response to intracolonic capsaicin. This suggests that an endogenous TRPV1 agonist is active in this model to evoke or maintain allodynia. One such potential agonist is the endogenous vanilloid/cannabinoid NADA. NADA is found in pharmacologically relevant concentrations throughout the CNS [23] and its synthesis is regu-

lated by neural activity [25]. NADA causes nocifensive behaviors and hyperalgesia when it is applied in the periphery [23,24] but its behavioral effect when injected into the spinal cord has not been explored. IT NADA did not cause nocifensive behaviors and had only a minor effect on punctate hyperalgesia (at 8 mN), however, it caused a robust stroking allodynia that was TRPV1-dependent. To our knowledge this is the first demonstration that an endogenous TRPV1 agonist causes allodynia when applied directly to the spinal cord further supporting the hypothesis that CNS TRPV1 receptors are critical for inflammatory allodynia [17].

NADA-mediated stroking allodynia was inhibited by the NKCC1 inhibitor, BUM, suggesting a role for NKCC1 in this effect. In NKCC1 knockout mice, hindpaw inflammation-mediated punctate hyperalgesia (von Frey) is preserved but stroking allodynia (cotton bud) is absent [8]. Our current observations with NADA and BUM are consistent with these findings from NKCC1 knockout mice insofar as stroking (or dynamic) allodynia appears to be the modality most affected in the hindpaw. It is unclear why punctate hyperalgesia and/or allodynia are not affected, however, dynamic allodynia has been suggested to be reflective of an interaction between Aβ- and C-fibers in the CNS following a sensitizing stimulus in experimental pain or in clinical pain in humans [31,32]. Mechanisms for increasing the activity of NKCC1 in primary afferent terminals have not been identified. In other CNS areas stimuli such as mGluR agonists and calcium-sensitive kinases are known to increase NKCC1 phosphorylation and activity [33]. TRPV1 activation stimulates extracellular regulated kinase 1&2 (ERK) [34] and calcium/calmodulin-dependent kinase II α (CaMKIIα) [35] activity both of which are kinases known to regulate NKCC1 activity [33,36]. Both ERK [18] and CaMKIIα [37] are involved in referred hyperalgesia evoked by intracolonic capsaicin hence, an intriguing hypothesis is that IT NADA activates ERK and/or CaMKIIα leading to an increase in NKCC1 activity causing touch evoked pain. On the other hand, it is possible that spinal TRPV1 activation mediates allodynia by regulating GABAergic neurotransmission. In this regard, it has been shown that dorsal horn TRPV1 stimulation leads to a large increase in GABAergic network activity [38] which may be an alternative explanation for the role of NKCC1 in allodynia mediated by NADA. Clearly more work is needed to better understand the potential interplay between NKCC1 and TRPV1 and its role in allodynia; however, the anti-allodynic effects TRPV1 antagonists and NKCC1 inhibitors on the spinal level indicate that this might be a fruitful avenue for combined therapeutics.

Conclusion

We have demonstrated that spinal NKCC1 blockade and spinal TRPV1 antagonism attenuates referred allodynia in response to a painful visceral stimulus. Moreover, we have shown that the endogenous TRPV1 agonist NADA, applied spinally, evokes stroking allodynia in the hindpaw that is TRPV1-dependent and inhibited by the NKCC1 blocker BUM. These findings further implicate NKCC1 in inflammatory allodynia and suggest that spinal TRPV1 receptors might be involved in regulating NKCC1 activity in nociceptive primary afferents.

Methods

Animals

Male C57BL6 mice between 20–25 g were used for all experiments. Experiments were in accordance with the Canadian Counsel on Animal Care (CCAC) and the International Association for the Study of Pain (IASP) guidelines for the care and use of experimental animals. All protocols were reviewed and approved by the McGill University Animal Care Committee.

Behavioral Testing and Statistical Analysis

Mice were habituated to a Plexiglass testing box (10 × 10 × 8 cm) for 1–2 hours prior to testing. The model of referred allodynia has been described previously [19], briefly, just prior to application of CAP, petroleum jelly (Vaseline) was applied to the peri-anal area to avoid the stimulation of somatic areas by contact with the capsaicin. A total of 0.05 ml of 0.1% capsaicin solution was slowly injected via a fine cannula with a rounded tip (external diameter 0.61 mm), gently introduced 4 cm into the colon via the anus. Capsaicin (0.1%, Tocris, Ellisville, MO) was dissolved in 10% ethanol, 10% Tween 80 and 80% saline. Bumetanide (BUM; Sigma, St. Louis, MO), AMG 9810 (Tocris) and n-arachidonoyl- dopamine (NADA, Tocris) were dissolved in artificial cerebrospinal fluid (aCSF) vehicle. The aCSF vehicle was comprised of (in mM) 1.3 CaCl2 2H2O, 2.6 KCl, 0.9 MgCl, 21.0 NaHCO3, 2.5 Na2HPO47H2O, 125.0 NaCl, and 3.5 dextrose (pH 7.2–7.4). Intrathecal (IT) injections of BUM, AMG 9801 and NADA were made in 5 μl aCSF on lightly anesthetized mice by lumbar puncture at the L4–L5 level with a 30-gauge needle on a 50 μl Hamilton needle [39]. Referred visceral hyperalgesia was tested on the abdomen with calibrated von Frey filaments (1mN & 32 mN) at various time-points before or after intracolonic capsaicin treatment. Percent response frequency was calculated from the number of responses to 10 stimulations on the abdomen. Each filament was applied for approximately 1–2 s, with an inter-stimulus interval of 2–5 s. The appearance of any of the following behaviors on application of a filament to the abdomen was considered a withdrawal response: (i) sharp retraction of the abdomen, (ii) immediate licking or scratching of site of application of fila-

ment, or (iii) jumping. For experiments with NADA, mice were habituated as above and tested on the hindpaw with von Frey filaments of 1, 4, 8 and 16 mN using the same protocol as for abdominal testing. Mice were also tested for stroking allodynia by gently brushing the plantar surface of the hindpaw 10 times with a 5 sec inter-stimulus interval with a cotton bud (Q-tip). The following reactions were considered a withdrawal response to the cotton bud: (i) sharp retraction of hindpaw, (ii) immediate licking or scratching of site of application of the cotton bud, or (iii) jumping. Mice were used for only one experimental procedure and were humanely killed by anesthesia overdose immediately after testing. All testing was performed blind to condition and treatment. Data are shown as mean ± SEM and all analyses involved one or two way anova (indicated in figure captions) with bonferroni post-tests, where $p < 0.05$ was considered significant.

Abbreviations

BUM, bumetanide; CaMKIIα, calcium/calmodulin-dependent kinase II α; DRR, dorsal root reflex; ERK, extracellular regulated kinase; KCC2, K^+, Cl^- co-transporter; NADA, narachidonoyl- dopamine; NKCC1, Na^+, K^+, $2Cl^-$ co-transporter type 1; PAD, primary afferent depolarization; TRPV1, transient receptor potential vanilloid type 1.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

FC and TJP conceived the experiments; FC, MHP and TJP designed experiments; MHP, TJP and JME performed the experiments; TJP, MHP and FC wrote the manuscript. All authors read and approved the final manuscript.

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The role of cation-dependent chloride transporters in neuropathic pain following spinal cord injury

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Abstract

Background: Altered Cl^- homeostasis and GABAergic function are associated with nociceptive input hypersensitivity. This study investigated the role of two major intracellular Cl^- regulatory proteins, Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and K^+-Cl^- cotransporter 2 (KCC2), in neuropathic pain following spinal cord injury (SCI).

Results: Sprague-Dawley rats underwent a contusive SCI at T9 using the MASCIS impactor. The rats developed hyperalgesia between days 21 and 42 post-SCI. Thermal hyperalgesia (TH) was determined by a decrease in hindpaw thermal withdrawal latency time (WLT) between days 21 and 42 post-SCI. Rats with TH were then treated with either vehicle (saline containing 0.25% NaOH) or NKCC1 inhibitor bumetanide (BU, 30 mg/kg, i.p.) in vehicle. TH was then re-measured at 1 h post-injection. Administration of BU significantly increased the mean WLT in rats ($p < 0.05$). The group administered with the vehicle alone showed no anti-hyperalgesic effects. Moreover, an increase in NKCC1 protein expression occurred in the lesion epicenter of the spinal cord during day 2–14 post-SCI and peaked on day 14 post-SCI ($p < 0.05$). Concurrently, a down-regulation of KCC2 protein was detected during day 2–14 post-SCI. The rats with TH exhibited a sustained loss of KCC2 protein during post-SCI days 21–42. No significant changes of these proteins were detected in the rostral region of the spinal cord.

Conclusion: Taken together, expression of NKCC1 and KCC2 proteins was differentially altered following SCI. The anti-hyperalgesic effect of NKCC1 inhibition suggests that normal or elevated NKCC1 function and loss of KCC2 function play a role in the development and maintenance of SCI-induced neuropathic pain.

Background

Spinal cord injury (SCI) and subsequent neuropathic pain can result in devastating motor and sensory deficits. Chronic neuropathic pain frequently develops following SCI and affects up to 70% of SCI patients clinically [1]. Effective analgesic therapy has been hampered by the lack of knowledge about the mechanisms underlying post-SCI neuropathic pain.

The GABAergic system plays an important role in spinal nociceptive processing. GABA receptors are found on pre- and post-synaptic sites of primary afferent terminals, as well as interneurons in laminae I-IV in the spinal cord dorsal horn [2]. GABAergic interneurons in the dorsal horn are important for nociceptive attenuation [3,4]. Subarachnoid implantation of GABA-producing neuronal cells in rats attenuates allodynia and hyperalgesia following excitotoxic injury [5]. Furthermore, administration of the $GABA_A$ receptor agonist muscimol prevents long-lasting potentiation of hyperalgesia following peripheral nerve injury [6]. However, the mechanism underlying the derangement of the GABAergic system during neuropathic pain state is unknown.

Normal GABAergic function is critically dependent on cation-chloride cotransporter activity, specifically inwardly directed Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and outwardly directed K^+-Cl^- cotransporter 2 (KCC2) [7-10]. Both NKCC1 and KCC2 are expressed in spinal cords and function to regulate intracellular Cl^- concentration. Increasing evidence suggests that changes of the transporter expression play a role in inflammatory or neuropathic pain [3,4,11,12]. Elevation of intracellular Cl^- can lead to GABAergic hypersensitivity by reversing both the Cl^- equilibrium potential (E_{Cl}) and the normal inhibitory action of GABA. However, it remains unknown whether NKCC1 and KCC2 play a role in chronic hyperalgesia following SCI.

In the current study, a contusive SCI at T9 was induced in adult male rats using the MASCIS impactor. Inhibition of NKCC1 with its potent antagonist bumetanide (BU) had an anti-hyperalgesic effect in rats with chronic neuropathic pain following SCI. Moreover, transient increase in NKCC1 protein and down-regulation of KCC2 expression were detected in the spinal cord following SCI. The results imply that these Cl^- transporter proteins may be a potential target for the development of analgesics following SCI.

Results

Anti-hyperalgesic effects of bumetanide

In order to assess the role of ion transporters in SCI-mediated hyperalgesia, it is important to verify that all animals experienced a similar degree of injury and exhibited similar locomotor function recovery prior to anti-hyperalgesic tests. Therefore, animals were randomly divided into one of two groups. In both group 1 and group 2, BBB scores showed classic locomotor function impairment after SCI

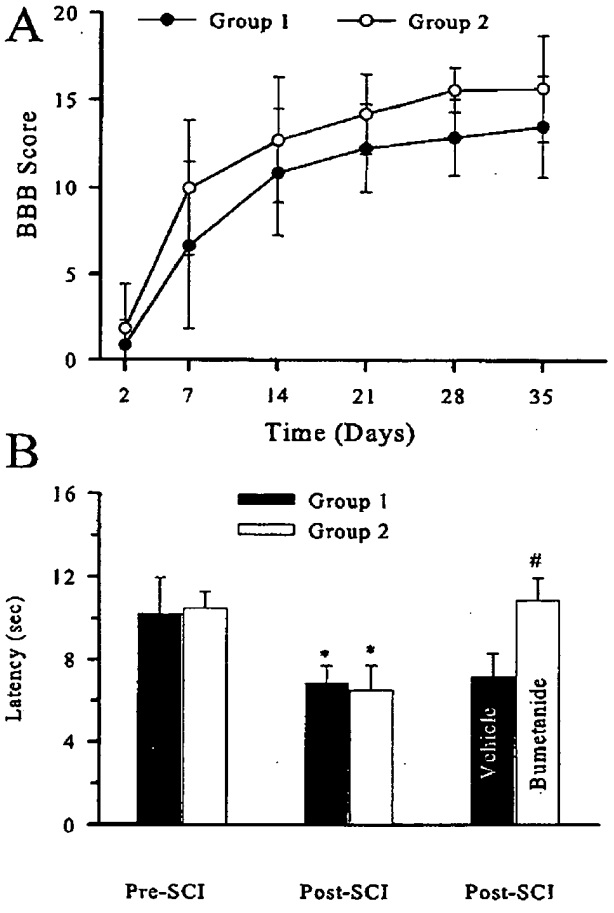


Figure 1

Anti-hyperalgesic effects of bumetanide. A. No difference in locomotor function following SCI in vehicle control and drug treatment groups. Animals were randomly divided into one of two groups (group 1 and 2). Locomotor function was monitored and Basso, Beattie, and Bresnahan (BBB) scores recorded on post-SCI days 2–42 in these groups. BBB scores recovered with time, and reached 13.5–15.7 by day 42 post-SCI. There were no significant differences in BBB scores between group 1 and group 2. B. Effects of Bumetanide on TH. TH was measured and the mean withdrawal latency time (WLT) recorded. Group 1 received vehicle (0.25% NaOH in saline, i.p.) as controls (n = 4) and group 2 received Bumetanide (BU 30 mg/kg, i.p., n = 8). After 1 h of treatment, TH was re-measured and the mean WLT recorded. Data are mean ± SD. * $p < 0.05$ vs. pre-SCI. # $p < 0.05$ vs. vehicle.

(Figure 1A). BBB scores recovered with time, and reached 13.5–15.7 by day 42 post-SCI. There were no significant differences in BBB scores between group 1 and group 2 (Figure 1A).

In the experiments to test anti-hyperalgesic effects of bumetanide, group 1 served as the vehicle control and group 2 was treated with bumetanide. Mean withdrawal latency time (WLT) was 10.5 ± 1.7 s prior to SCI. In vehicle-treated animals (group 1), the mean WLT on 21–42 days post-SCI was reduced to 6.9 ± 0.8 s ($p < 0.05$, Figure 1B), which is consistent with our previous findings with SCI-induced TH [13]. Subsequent injection of the vehicle alone had no effects on the level of SCI-induced TH (7.2 ± 1.2 s). In contrast, following the administration of BU (30 mg/kg, i.p, group 2), there was a significant increase in mean WLT (from 6.5 ± 1.2 to 10.9 ± 1.1 s, $p < 0.001$), demonstrating an anti-hyperalgesic effect of BU. Moreover, to evaluate for potential non-specific analgesic drug effects, BU and vehicle controls were also administered in rats which did not demonstrate TH. No significant changes in mean WLT were observed in these animals (9.2 ± 1.8 s in vehicle-treated vs. 11.0 ± 0.74 s in BU-treated rats, $p > 0.05$). A lower dosage of BU (15 mg/kg, i.p.) gave rise to a similar anti-hyperalgesic effect as was observed with the higher dosage (WLT of 10.9 ± 2.8 vs. 7.2 ± 1.2 s in vehicle-treated rats, $p < 0.05$, $n = 4$).

Expression of NKCC1 and KCC2 following SCI

We investigated changes in NKCC1 protein levels in the epicenter of spinal cord tissue harvested at 2, 7, and 14 days post-SCI. A low level of NKCC1 protein was expressed in the spinal cord tissue at baseline (Figure 2A). βIII-tubulin was probed on the same blot to serve as control. Densitometric analysis of the NKCC1/βIII-tubulin band ratio intensities is shown in Figure 2A. NKCC1 was elevated on day 7 post-SCI and increased by ~60% on day 14 post-SCI ($p < 0.05$). In contrast, KCC2 protein was decreased on 2 and 7 days post-SCI and significantly fell by ~40% on day 14 post-SCI ($p < 0.05$, Figure 2B). However, no significant changes of βIII-tubulin were detected in the same sample. This data indicate a significant increase in the expression of NKCC1 and, conversely, a decrease in KCC2 expression at the epicenter on day 14 post-SCI (prior to the chronic phase of post-SCI neuropathic hyperalgesia).

To further establish that changes in NKCC1 and KCC2 are specific to the lesion epicenter of the spinal cord in response to SCI, we also examined spinal cord tissue rostral to the SCI injury epicenter. As shown in Figure 3A, NKCC1 protein level was low in sham control animals and tended to increase on days 2–14 post-SCI, but did not reach statistical significance. There were also no significant changes in KCC2 protein expression in rostral spinal cords on day 2–14 post-SCI (Figure 3B).

Changes of NKCC1 and KCC2 in hyperalgesia rats following SCI

We examined changes in NKCC1 and KCC2 protein levels in the contusion epicenter of spinal cord during days 21–42 post-SCI, when it was possible to determine the presence of chronic TH. As shown in Figure 4, a moderate reduction of NKCC1 occurred on days 21–35 post-SCI.

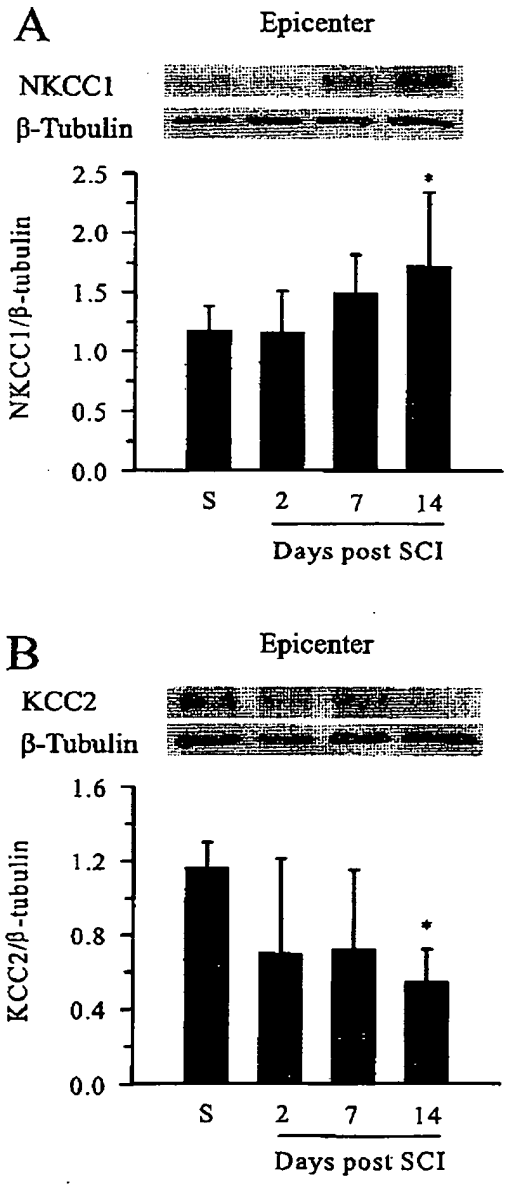
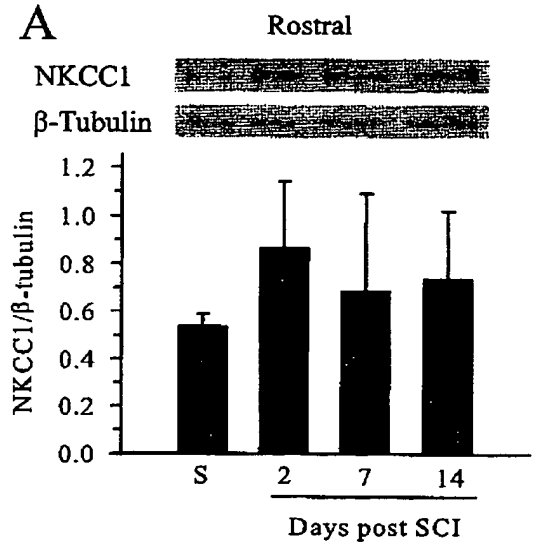


Figure 2



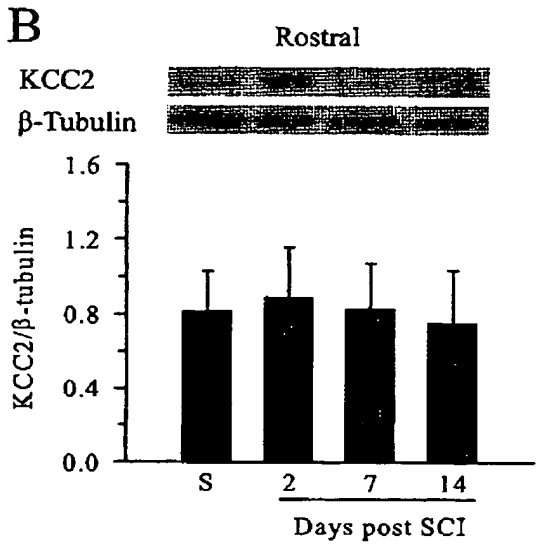


Figure 3
Expression of NKCC1 and KCC2 in rostral spinal cord tissues after SCI. A. Samples were prepared from spinal cord tissue rostral to the injury epicenter at 2, 7, and 14 days after SCI. Top panel, the blot was probed with anti-NKCC1 antibody (1:4000) and anti-βIII-tubulin antibody (1:5000). Lower panel, summary of densitometry data. Data are mean ± SD, n = 4. B. KCC2 expression post-SCI. Top panel, blots were probed with anti-KCC2 antibody (1:500) and anti-βIII-tubulin antibody. Lower panel, summary of densitometry data. Data are mean ± SD, n = 6.

Figure 2
Early changes in expression of NKCC1 and KCC2 proteins in epicenter spinal cord tissues following SCI. A. NKCC1 protein expression in epicenter spinal cord tissues at 2, 7, and 14 days post-SCI. Sham (S) samples were acquired from animals subject to laminectomies without subsequent spinal cord contusion. Top panel, the blot was probed with anti-NKCC1 antibody (1:4000) and anti-βIII-tubulin antibody (1:5000). Lower panel, Densitometric analysis of the ratio of NKCC1/βIII-tubulin band intensity. Data were mean ± SD, n = 6. * $p < 0.05$ vs. Sham. B. Decrease in expression of KCC2 following SCI. Top panel, the blots were probed with anti-KCC2 antibody (1:500) and anti-βIII-tubulin monoclonal antibody (1:5000). Lower panel, summary of densitometry data for the ratio of KCC2/βIII-tubulin. Data were mean ± SD, n = 3. * $p < 0.05$ vs. S.

KCC2 proteins remained down-regulated during days 21 and 35 post-SCI. Interestingly, in rats exhibiting no TH, both NKCC1 and KCC2 proteins remained unchanged as compared to sham. In contrast, TH rats displayed a sustained loss of KCC2 protein, with expression of KCC2 observed to be 34% and 40% of sham in lesion epicenter tissue at days 28 and 35 post-SCI, respectively. Injured spinal cord tissues develop neurodegeneration and severe glial scar formation after post-SCI day 20 [14]. Therefore, due to neuronal death and astrogliosis, no routine loading control markers (such as β-tubulin III for neurons and GFAP for glia) are appropriate for a quantitative immunoblotting analysis. In Figure 4, various changes of GFAP were illustrated, reflecting astrogliosis. As a result of this obstacle, no statistical analysis was performed. However, at least in the post-SCI day 28 samples, when higher levels of GFAP were detected in the spinal cord tissues in animals with neuropathic pain, the loss of KCC2 protein remained, which strongly suggests that the decrease in KCC2 expression is unlikely due to sample loading errors (insufficient protein loading).

Discussion

NKCC1 and KCC2 in hyperalgesia

The inhibitory action of GABA is a critical component of numerous neuronal circuits. It has been proposed that large, myelinated Aβ-fibers could antagonize nociceptive primary afferent inputs to the dorsal horn through inhibitory mechanisms mediated by interneurons. These interneurons release GABA which activates $GABA_A$ receptors on primary afferent terminals and produces primary afferent depolarization (PAD). PAD shunts the magnitude of incoming action potentials and decreases excitatory amino release at the primary afferent central terminals [3,4]. However, under inflamed conditions, PAD may be enhanced such that it leads to excessive depolarization of

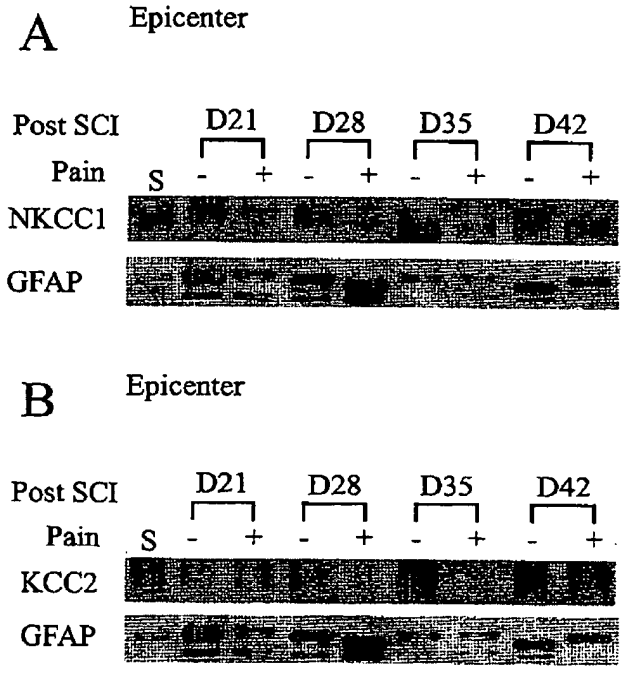


Figure 4
Changes in NKCC1 and KCC2 expression in non-hyperalgesic and hyperalgesic rats. NKCC1 protein expression in the injury epicenter spinal cord tissues at 21, 28, 35, and 42 days post-SCI. Sham (S) samples were acquired from animals subject to laminectomies without subsequent spinal cord contusion. Top panel, the blot was probed with anti-NKCC1 antibody and anti-GFAP (glial fibrillary acidic protein) antibody. Lower panel, the blot was probed with anti-KCC2 antibody and anti-GFAP antibody. Hyperalgesic rats (labeled as pain) were identified with the thermal hyperalgesia withdrawal latency test as described above. (+): hyperalgesia; (-): without hyperalgesia. n = 2.

Aδ- and C-fibers above their thresholds for action potential generation.

Any injury-induced modification of this inhibitory action has the potential to alter the processing of nociceptive information in the spinal dorsal horn. Therefore, the generation of Cl^--dependent $GABA_A$ receptor response is critically dependent on the activity of NKCC1 and KCC2. NKCC1 knockout mice ($NKCC1^{-/-}$) lack the $GABA_A$ receptor-mediated anion outward flux current [11]. $NKCC1^{-/-}$ mice have deficits in thermal nociceptive threshoulds and display a decrease in Aβ-fiber-mediated touch-evoked allodynia following capsaicin injection [11]. Moreover, the NKCC1 antagonist bumetanide inhibits itch and flare responses to histamine in human skin, and attenuates phase I and II behavioral responses in the formalin model of tissue injury-induced pain [15]. After intra-colonic capsaicin injection in mice, NKCC1 plasma membrane expression and phosphorylation are increased in the dorsal spinal cord, although it is unknown whether it is accompanied with KCC2 down-regulation [16]. Inhibition of NKCC1 and TRPV1 attenuates capsaicin-induced allodynia [17]. These results suggest that NKCC1 plays a role in the initiation of hyperalgesia. On the other hand, intraplantar formalin stimulation triggers a significant decrease in KCC2 protein expression without changes in NKCC1 in the rat spinal cord [18]. Taken together, these studies suggest that alteration of Cl^- homeostasis by changes in NKCC1 and/or KCC2 may contribute to hyperalgesia development.

NKCC1 and KCC2 in neuropathic pain

The current study examined the role of NKCC1 and KCC2 in chronic neuropathic pain following a contusion SCI. Immunoblotting showed a significant transient up-regulation of NKCC1 protein in the lesion epicenter of the rat spinal cord on day 14 post-SCI. This was accompanied by a concurrent down-regulation of KCC2 protein. Moreover, inhibition of NKCC1 with its potent antagonist BU significantly reduced pain behavior in rats. These results imply that altered NKCC1 and KCC2 expression may lead to GABAergic derangement and contribute to the induction and maintenance of the chronic neuropathic pain following SCI. BU inhibits NKCC1, but also KCC and other anion transport processes (e.g., Cl^-/HCO_3^- exchange, Cl^- channels etc.) at higher concentrations. However, the concentration of bumetanide (5 or 10 μM) is relatively specific for NKCC1 [19].

Partial nerve injury (induced by a sciatic cuff) disrupted anion homeostasis in lamina I neurons and shifted the normally inhibitory synaptic currents to excitatory, thereby increasing lamina I neuronal excitability *in vitro* [20]. The nerve injury was associated with a decrease in KCC2 mRNA and protein levels. Moreover, inhibition of KCC2 activity *in vivo* reduces mechanical and thermal nociceptive thresholds in control, and uninjured animals [20]. In addition, *in vivo* axonal injury causes a reduction of KCC2 mRNA in motor neurons and results in an increased intracellular Cl^- concentration and, consequently, $GABA_A$ receptor-mediated excitatory responses [21]. A recent report shows that this transient down-regulation of KCC2 [22] and the early pain behavior depends on activation of TrkB receptor via BDNF in nerve injury [12].

Post-translational modulation of KCC2 appears to be involved in the down-regulation of KCC2 protein. Exposing hippocampal neurons to H_2O_2 leads to a rapid dephosphorylation of KCC2 protein and a subsequent down-regulation of KCC2 protein [23]. A loss of tyrosine

phosphorylation of KCC2 and a reduction of surface-expression of KCC2 in the plasma membrane is largely attributable to tyrosine phosphatases [23]. In our current study, reduction of KCC2 protein was detected in the epicenter region of the spinal cord on day 14–42 following SCI. It remains to be determined whether altered KCC2 phosphorylation is responsible for translocation between the plasma membrane and intracellular compartments in SCI. It is also unknown whether changes of KCC2 oligomerization play a role in neuropathic pain following SCI.

The significance of changes of NKCC1 and KCC2 proteins following SCI is not clear. Chloride homeostasis through the function of ionotropic GABA receptors has emerged as one important mechanism in the development of immature neurons [8]. NKCC1 phosphorylation stimulates neurite growth of injured adult sensory neurons [24]. Therefore, elevation of NKCC1 and down-regulation of KCC2 in lesion epicenter may reflect the cellular repair responses following SCI. However, the subsequent alteration of Cl^- homeostasis and GABAergic function causes neuropathic pain.

Conclusion

In summary, we investigated the roles of two major intracellular Cl^- regulatory proteins, NKCC1 and KCC2, in chronic neuropathic pain following contusive SCI in the rat model. Contusive SCI caused a decrease in mean hind-paw thermal WLT in rats during the 21–42 day period post-SCI. Inhibition of NKCC1 with its potent antagonist BU significantly reduced pain behavior in rats. Immunoblotting showed a significant transient up-regulation of NKCC1 protein in epicenter of the rat spinal cord on day 14 post-SCI, which was accompanied by a concurrent down-regulation of KCC2 protein. Sustained reduction of KCC2 protein was prominent in epicenter of the spinal cord in TH rats. These results imply that altered NKCC1 and KCC2 expression may contribute to the induction and maintenance of the chronic neuropathic pain following SCI.

Materials and methods

Contusion SCI

All animal procedures used in this study were conducted in strict compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the University of Wisconsin Center for Health Sciences Research Animal Care Committee.

Adult male Sprague-Dawley rats (250–300 g) were anesthetized with gaseous isoflurane in oxygen (5% for induction, 3% for maintenance) throughout the duration of surgery. A T9 laminectomy was performed without disrupting the dura mater. Stabilization vertebral clamps were placed at T8 and T10 to the MASCIS Impactor (WM Keck Center for Collaborative Neuroscience, Rutgers University, Piscataway, NJ; Model II) and the spinal cord injured by releasing a 10 g rod (2.5 mm diameter) from a height of 12.5 mm as described previously [13]. Bupivacaine (Sensorcaine-MPF 0.25%, 0.20 ml) was administered subcutaneously as a local anesthetic. Throughout the procedure, body temperature was maintained at 37°C with a constant temperature heating pad. The animals were then returned to their cages after recovering from anesthesia. Animals underwent daily manual bladder expression until bladder control was re-established and each animal received Cefalexin antibiotic (0.10 mL; 330 mg/mL in saline) for 7 days post-injury. Six rats were designated as sham controls and underwent T9 laminectomy under anesthesia without the subsequent spinal cord contusion.

Locomotor function

The functional neurological deficits due to the SCI were assessed by behavioral analysis using the Basso, Beattie, and Bresnahan (BBB) open-field locomotor test [25-27]. Animals were observed individually in an open field testing area consisting of a plastic wading pool. BBB scores were measured before injury (baseline) and on post-injury days 2, 7, 14, 21, 28, 35, and 42 from video recordings of 4 minutes per animal. BBB scores ranged from 0 (no hind limb movement) to 21 (normal movement – i.e. coordinated gait with parallel paw placement).

Thermal withdrawal latency time

Thermal hyperalgesia (TH) was assessed using the hind paw withdrawal test to a thermal noxious stimulus [28]. TH is a sensitive and reproducible behavioral test of neuropathic pain, which is typically exhibited in the animals beginning approximately 21 days following SCI [13,29-31]. Rats were placed inside the Plexiglas apparatus (Plantar Test, Ugo Basile, Italy) and allowed to acclimatize. A noxious heat stimulus was applied to the plantar surface of the hind paw and the withdrawal latency time (WLT) recorded. Results of each test are expressed in seconds (s) as the mean of six withdrawal latencies (three from each hind paw). Animals were tested at different time points in order to avoid a training effect. Specifically, TH testing was performed prior to injury (baseline) and on post-injury days 21, 28, 35, and 42. A decrease in WLT of $\geq$ 2 sec from baseline is considered to be significant [31]. Onset of TH between days 21–42 post-injury occurred in nearly half of the injured animals, representing the chronic phase of post-SCI neuropathic hyperalgesia [13].

Drug preparation

The NKCC1 antagonist bumetanide (BU) stock was prepared in 10 mg/mL of saline containing 0.25% NaOH.

Control vehicle was prepared as 0.25% NaOH in saline. Control vehicle or BU (30 mg/kg) was randomly administered intraperitoneal (i.p.) into two groups (group 1, group 2) of rats exhibiting neuropathic hyperalgesia on post-SCI 21–42 days. TH was re-measured 1 h post-injection.

Spinal cord harvesting

Spinal cords were harvested as two 7–10 mm sections: the epicenter of the contusion and rostral to the contusive lesion. The sections were immediately submerged in liquid nitrogen and stored at -80°C. Spinal cord samples were harvested from animals prior to onset of the chronic phase of neuropathic hyperalgesia (days 2, 7, and 14 following SCI) and on days 21, 28, 35, and 42 following SCI, when neuropathic hyperalgesia was developed. The spinal cords of sham control animals were harvested on day 2 post-surgery.

Sample preparation and western blotting

The harvested spinal cord segments were homogenized in anti-phosphatase buffer (pH 7.4, mmol/L: 145 NaCl, 1.8 NaH_2PO_4, 8.6 Na_2HPO_4, 100 NaF, 10 $Na_4P_2O_7$, 2 Na_3VO_4, 2 EDTA) and protease inhibitors as described previously [32]. The homogenate was centrifuged at 7000 rpm for 15 min at 4°C. The supernatant was retained and protein content of the supernatant was determined by the BCA protein assay (Pierce; Rockford, Ill). Protein samples (20 μg/lane) and pre-stained molecular mass markers (Bio-Rad; Hercules, CA) were denatured in SDS sample buffer. The samples were then electrophoretically separated on 6% SDS gels, and the resolved proteins were electrophoretically transferred to a PVDF membrane. The blots were incubated in 7.5% nonfat dry milk in Tris-buffered saline (TBS) overnight at 4°C, and then incubated for 1 h with a primary antibody. The blots were rinsed with TBS and incubated with horseradish peroxidase-conjugated secondary IgG for 1 h. Bound antibody was visualized using the enhanced chemiluminescence assay (ECL, Amersham Corp; Piscataway, NJ). Monocolonal antibody against NKCC1 (T4, 1:4000; Developmental Studies Hybridoma Bank; Iowa City, IA), and anti-KCC2 polyclonal antibody (1:500; Millipore, Billerica, MA) were used for detection of NKCC1 and KCC2, respectively. Anti-βIII-tubulin monoclonal antibody was used as a loading control (Promega; Madison, WI). Densitometric measurement of each protein band was performed with Un-Scan-It software (Silk Scientific; Orem, UT) and average pixel intensity was recorded.

KCC2 exists in monomeric and oligomeric structures [33]. In the current study, all protein samples were electrophoretically separated on 6% SDS gels in the presence of sulthydryl-reducing agent β-mercaptoethanol (5%). As reported by Blaesse et al [33], β-mercaptoethanol results in the disassembly of the three oligomeric structures of KCC2 oligomers. Therefore, we mainly analyzed changes of KCC2 monomers in the current study (~130 kDa).

Statistical analysis

Comparisons between groups were made by student t-test and Mann-Whitney rank sum test where necessary (SigmaStat, Systat Software; Point Richmond, CA). A $p < 0.05$ was considered a statistically significant difference.

Abbreviations

BBB: Basso, Beattie, and Bresnahan open-field locomoter test; Bu: Bumetanide; GFAP: glial fibrillary acidic protein; KCC2: K^+-Cl^- cotransporter 2; NKCC1: Na^+-K^+-Cl^- cotransporter 1; PAD: primary afferent depolarization; SCI: spinal cord injury; TH: thermal hyperalgesia; WLT: withdrawal latency time.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GM and SR were responsible for animal surgery, pain data collection, and analysis. SC was responsible for all immunoblotting experiments. GM, SR, DS, and DR were involved in experimental design. DS, CB, SC, and DR were involved in manuscript writing. All authors read and approved the final manuscript.

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